

NEUROLOGICAL ABNORMALITIES

IN

PERNICIOUS ANEMIA

METABOLIC MECHANISMS

HENRY OLDERS  
MCGILL UNIVERSITY

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# **NEUROLOGICAL ABNORMALITIES IN PERNICIOUS ANEMIA: METABOLIC MECHANISMS**

## ***Introduction***

Pernicious anemia is a nutritional disorder characterized by a deficiency of cobalamin, (also called vitamin B12), which is due to lack of intrinsic factor. Clinical manifestations of this cobalamin deficiency fall into two broad classes: (a) megaloblastic anemia; and (b) a variety of neurological symptoms. It should be pointed out that pernicious anemia is only one of a number of diseases which have similar clinical features resulting from a cobalamin deficiency. Other pathological states include cobalamin malabsorption and the postoperative states resulting from (1) ileal resection (cobalamin is absorbed in the ileum) and (2) gastrectomy (which is similar to pernicious anemia; in that no intrinsic factor is produced), parasites, lack of cobalamin in the diet, or increased nutritional requirements because of pregnancy, neoplastic disease, etc. Most of what is known about deficiencies of cobalamin in humans, however, has been learned from studies of pernicious anemia (PA) patients.

## ***Neurological Symptoms of Cobalamin Deficiency***

The classical neurologic syndrome of pernicious anemia consists of symmetrical paresthesias in the feet and hands, with associated disturbances of vibratory sense and proprioception, progressing to spastic ataxia (Beck, 1975). These symptoms result from degenerative changes in the dorsal and lateral columns of the spinal cord, termed "subacute combined degeneration" Other clinical signs may include cerebral manifestations, irritability, somnolence, perversions of taste, smell, and vision, and occasionally optic atrophy. Smokers may present with the visual disorder of tobacco amblyopia, thought to be due to the cyanide in tobacco smoke.

Grossly, the spinal cord lesions in the dorsal and lateral columns appear shrunk and grayish-white. Under the microscope, myelin swelling is seen, followed by demyelination, and later by axonal degeneration. In some cases, foci of demyelination may also be found in the brain stem, optic nerves, and cerebral cortex (Robbins et al, 1976).

## ***Metabolic Roles of Vitamin B12***

In order to discuss the possible mechanisms of a cobalamin deficiency in bringing about the above neurological symptoms, it is first necessary to describe the known metabolic roles of this vitamin in man.

Although a number of cobalamin-dependent reactions have been identified in bacterial systems, only two reactions have been shown to require the vitamin in man (Stryer, 1975):

- A) conversion of methylmalonyl CoA to succinyl CoA
- B) conversion of homocysteine to methionine

In reaction A, the deoxyadenosylcobalamin form of the vitamin acts as a coenzyme of methylmalonyl mutase. The product, succinyl CoA, is a citric acid cycle intermediate, and thus this reaction is part of the catabolic breakdown of the amino acids methionine, threonine, isoleucine (all via propionyl CoA), and valine. In addition, the pathway from propionyl CoA to succinyl CoA via methylmalonyl CoA also participates in the oxidation of odd-numbered fatty acids.

In reaction B, the methylcobalamin form of cobalamin acts as a coenzyme for homocysteine transmethylase, the enzyme which catalyses the transfer of a methyl group from N5-methyl tetrahydrofolate (abbreviated methyl-THF) to homocysteine to form methionine. The methionine is subsequently activated by ATP to S-adenosylmethionine (SAM), a compound which is capable of transferring its methyl group to a variety of other substances. The resulting S-adenosylhomocysteine (SAH) is hydrolyzed to adenosine and homocysteine. This "activated methyl cycle" is responsible for donating methyl groups in a variety of synthesis reactions, including the conversion of phosphatidyl ethanolamine to phosphatidyl choline (lecithin). However, other mechanisms (which do not require cobalamin) exist for methylating homocysteine to methionine. Thus, it may be that the importance of cobalamin in this reaction is not the synthesis of homocysteine, but the conversion of methyl-THF to tetrahydrofolate (Beck, 1975).

### ***Proposed Roles of Cobalamin***

A number of hypotheses have been put forward regarding other roles for cobalamin in animal cells. These proposals are based on observations which may have other explanations:

Hypothesis 1: Cobalamin is required for the uptake of methyl-THF by cells (a membrane transport role). This is based on investigations of methyl-THF uptake by dividing lymphocytes (Das et al, 1970) and by bone marrow cells (Tisman et al, 1973). In both cases, uptake rates were subnormal in cells from PA patients, but normal in patients with folate deficiency.

Hypothesis 2: Cobalamin is involved in the metabolism of cyanide. It has been proposed that cobalamin, which has a high affinity for cyanide, plays a role in the excretion of cyanide as cyanocobalamin, and may also be required for the conversion of cyanide to thiocyanate (a major metabolic route for cyanide when it is present in toxic concentrations) (Wokes et al, 1955). Humans are exposed to cyanides from tobacco smoke, from fungi, industrial effluents, as well as from fruits, beans, and nuts. It is thought that some of this cyanide converts a proportion of the body's cobalamin supply into metabolically useless cyanocobalamin. While this would not affect normal individuals, it might worsen a pre-existing cobalamin deficiency. Alternatively, low cobalamin levels may compromise the body's capacity for detoxifying cyanide.

Hypothesis 3: Cobalamin affects the activity in cells of thymidylate synthetase (Haurani, 1973), the enzyme responsible for converting deoxyuridylate (dUMP) to thymidylate (dTMP), using N5,N10-methylene-THF as a cofactor. This proposal is based on observations that synthetase activity in stimulated lymphocytes of patients with cobalamin deficiency was much less than for normals or patients with folate deficiency.

## ***Metabolic Basis of Neurology of Cobalamin Deficiency***

It should be pointed out that the neurological abnormalities of cobalamin deficiency often share similarities with the neuropathies associated with other deficiencies or toxic states. These may provide clues to the metabolic changes involved. Of particular interest are the following:

- a) in "tobacco amblyopia", a disorder which appears to occur primarily in smokers, the visual disabilities are similar to the optic atrophy of cobalamin deficiency. In fact, this deficiency has been found in tobacco amblyopia (Heaton et al, 1958), and it has also been suggested that hydroxocobalamin is more effective therapeutically for this condition than cyanocobalamin (Chisolm et al, 1967). This supports the hypothesis relating cobalamin to the detoxification of cyanide from tobacco smoke.
- b) In West Africa and the West Indies, relatively high incidences of retrobulbar neuritis (inflammation of the optic nerve, between the globe of the eye and the optic foramen; sometimes found in PA patients) and of optic atrophy have been found in native populations who eat cassava, which is rich in cyanide (Montgomery, 1965).
- c) Congenital defects of the enzyme steps involved in the breakdown of homocysteine are known as homocystinuria and cystathioninuria. Both diseases are characterized by mental retardation (McGilvery, 1970). Since the "activated methyl cycle" is probably somewhat disrupted in these conditions due to high homocysteine concentrations, a mechanism similar to the effect of cobalamin deficiency on the conversion of homocysteine to methionine may be at work. In this connection, there have been observed three children with mental retardation, increased tendon reflexes, and impaired proprioception, but no megaloblastic anemia. Evidence of defective formation of methyl- and adenosylcobalamin was found in these children, who displayed severe homocystinuria, cystathioninuria, and methylmalonic aciduria, as well as low serum levels of methionine. Skin fibroblast cultures from these patients had low levels of homocysteine methyltransferase activity (Levy, 1970).
- d) Folate deficiency may adversely affect the nervous system. Folate deficiencies have been found in patients who have spinal cord and peripheral neurological signs that are indistinguishable from subacute combined degeneration (Freeman, 1976). Some inborn errors of folate metabolism manifest as mental retardation (Tauro et al, 1976). Lately there have been reports of folate-responsive neuropsychiatric disease in epileptic, neurological, psychiatric, and geriatric patients (Editorial, 1976; Reynolds, 1976 ). It is difficult to assign a casual relationship, since the illness itself may be responsible for a folate deficiency - from drugs, diet, anorexia, etc. It should also be pointed out that many patients with folate deficiency do not display any neuropathology. However, this is true also of cobalamin deficiency, and can perhaps be ascribed to differences in the tissues involved in either megaloblastosis or neuropathy, such as permanent vs. labile cell types; existence of the blood-brain barrier, and so on (Reynolds, 1976 ).
- e) The inborn error of metabolism "non-ketotic hyperglycinemia", characterized by excess glycine in the blood and CSF, has as its clinical

manifestation seizures, mental retardation, and early death. This disease is the result of a lesion in the "glycine cleavage system" which metabolizes glycine to donate a methylene group to tetrahydrofolate (MacKenzie, 1978).

The above evidence appears to implicate (very circumstantially) several different mechanisms which may contribute to the neuropathology of cobalamin deficiency:

- (1) folate metabolism, which is related to cobalamin via the homocysteine-to-methionine conversion (reaction B);
- (2) cyanide metabolism (via cyanocobalamin);
- (3) excess homocysteine or cystathionine (reaction B). Several other potential mechanisms can also be introduced at this point:
- (4) excess methylmalonic acid or propionic acid (reaction A);
- (5) insufficient synthesis of phosphatidyl choline (lecithin); and
- (6) defective methylation of ribosomal RNA. Each of these possibilities will now be examined.

### ***Folate Metabolism***

As described earlier, cobalamin participates as a coenzyme in the transfer of a methyl group from methyl-THF to homocysteine, a reaction which has tetrahydrofolate (THF) and methionine as its products.

Folic acid (pterin-paraaminobenzyl glutamic acid) is found mainly in nature in the conjugated form - that is, a chain of glutamate residues of varying length. Folate is hydrogenated by a two-step reduction in the liver to the THF form, which can bind one-carbon groups (methyl, methylene, formyl, formimino, or methenyl) to its N5 and/or N10 positions. Although folate is deconjugated in the intestine to the monoglutamate form (which is best absorbed), it becomes reconstituted in the target cell (Simkus, 1978). Folate is concentrated in the CSF to five times its serum concentration by the choroid plexus.

Normally, the predominant form of folate in serum and liver is methyl-THF, which is considered the storage form. Methylene-THF is formed from THF by obtaining the methylene group from glucose, serine, or glycine metabolism. The methylene-THF can participate in the synthesis of thymidylate from deoxy-uridylate, be reduced to methyl-THF in an essentially irreversible reaction, or be further oxidized to methenyl-THF. Methyl-THF, of course, participates in the synthesis of methionine.

Methenyl-THF can be converted to formyl-THF. Both of these species are necessary for the de novo synthesis of purines. If there is an excess of one-carbon fragments in the folate one-carbon pool, the "overflow pathway" converts formyl-THF to THF with the concurrent release of CO<sub>2</sub>.

In the metabolism of histidine, one of the carbons ends up in the one-carbon pool as methenyl-THF, via formimino-glutamate and formimino-THF. If insufficient folate is available to form formimino-THF, then the intermediate product formimino-glutamate will be excreted in the urine.

The "methyl trap" hypothesis has been put forward to explain the megaloblastic anemia seen in cobalamin deficiency. According to this hypothesis, reduced methyltransferase activity (reaction B) means that THF which gets converted to methyl-THF becomes "trapped" in this form, thus making some of the folate

stores unavailable, and thereby reducing those syntheses which depend on the folate one-carbon pool. In particular, the conversion of dUMP to dTMP by thymidylate synthetase, which requires methylene-THF, will be compromised. The resultant inability to synthesize DNA will result in megaloblastosis. Beck provides a good review of the evidence for and against the "methylfolate trap" hypothesis (Beck, 1975). One observation, not mentioned by him, which supports the line of reasoning of the hypothesis, is that thymidylate injections into a PA patient reversed the megaloblastosis (Cooper, 1978). Also in support of the hypothesis, it is argued that sometimes the neurological complications of folic-acid deficiency are indistinguishable from cobalamin deficiency. Also, the highest serum-folate levels (i.e. of methyl-THF) are found in those patients with neurological complications of cobalamin deficiency. Some arguments oppose this hypothesis; for example, there is poor correlation between the hematological and the neurological complications in cobalamin deficiency. Moreover, administration of folate to PA patients brings about remission of the anemia but simultaneously may lead to precipitation or aggravation of the neuropathy (Editorial, 1976).

Recent work by Das and Herbert reinforces the role of folate deficiency caused by the "methylfolate trap" in cobalamin deficiency. They found that thymidylate synthetase activity is decreased in cobalamin deficiency, not because of a lack of synthesis of the apoenzyme, but because of lack of 5,10-methylene-THF. (Das et al, 1977) Note that this probably contradicts any direct role of cobalamin in affecting the activity of this enzyme (hypothesis 3).

The methyl trap hypothesis is further supported by recent experiments on rats which show that the reduced net uptake of folate by tissues, which is found to occur in cobalamin deficiency is not due to a role of cobalamin in membrane transport of folate (see hypothesis 1 above). Rather, it was shown that initial folate uptake was normal in cobalamin deficient rats, and that the reduced net uptake over longer periods correlated with a decreased ability in the cobalamin-deficient animals to synthesize polyglutamate folates (Shane et al, 1977). In other words, the cobalamin deficiency results in increased levels of methyl-THF (by the "methyl trap") which causes less polyglutamate folates to be synthesized (since methyl-THF is a poor substrate for the synthetase which generates polyglutamates). Because tissues do not retain the methyl-THF as well as polyglutamate forms, the end result is the decreased tissue folate levels seen by other researchers

This conclusion is bolstered by data obtained by Perry and his coworkers (Perry et al, 1977) from patients with untreated PA. They found an accumulation of folates with short glutamate chains, but an overall decrease of polyglutamates in these patients, compared to normals. In comparison, patients with folate deficiencies showed an overall fall in the amount of folate, with a concomitant decrease in the relative amount of short chain glutamates, when compared to normals.

Also working with rats, other researchers have found decreases in liver folate levels, accompanied by decreases in the rate of histidine oxidation (which requires THF; see above) for animals which were deficient in cobalamin and methionine (Chiau F et al, 1977).

On balance, the available evidence seems to support the view that a cobalamin deficiency results in disturbances to folate metabolism, via the mechanism of the

"methyl trap". The reduction in available folate probably acts to reduce thymidylate synthetase activity, which accounts for the megaloblastosis via a mechanism identical to that for folate deficiency (i.e. by prevention of DNA synthesis). Effects on synthetase activity should not cause neurological symptoms, since cell replication is not taking place in the CNS. We can, however, examine other possible effects of the disturbed folate metabolism to bring about neuropathy. It must be stressed that mechanisms based on altered folate metabolism need not exactly parallel the results of folate deficiency, since the biochemical effects of the two are different. For example, cobalamin deficiency probably leads to high methyl-THF in serum, but not in tissue. It may also lead to low levels of long-chain glutamate forms of folate, whereas folate deficiency results in low levels of folate, both long and short chain forms, in all tissues. If it is assumed that short-chain folates have different activities from the long-chain forms in various pathways (perhaps due to different tissue distributions), then it is possible that neurological symptoms can result from one or both of the following: 1. excess levels of glycine (which requires folate for its metabolism). Note that congenital hyperglycinemia produces mental retardation. 2. defective RNA synthesis, due to decreased activity in the de novo pathway for purine synthesis, possibly resulting in altered synthesis of membrane proteins or enzymes (for example, those enzymes used for turnover of myelin components). The existence of these hypotheses should not lead to the assumption that all neurological symptoms seen in cobalamin deficiency are the result of altered folate metabolism. Indeed, it seems likely that in most cases, other mechanisms overshadow entirely the possible effects of folate on the CNS. The above hypotheses are useful primarily for explaining the occasional neurological symptoms seen in folate deficiency.

### ***Cyanide Metabolism***

There is considerable evidence, as pointed out above, that cyanide is implicated in two of the neurological symptoms sometimes seen in cobalamin-deficient cases: (1) amblyopia and (2) retrobulbar neuritis. It is hypothesized that cobalamin will combine with cyanide to form cyanocobalamin, which is then excreted, thus ridding the body of toxic cyanide. It is not clear whether the neuropathy seen in tobacco smokers who may also have a cobalamin deficiency, should be ascribed to (a) the direct effects of cyanide intoxication resulting from a reduced capability to detoxify cyanide, or (b) mechanisms caused by a cobalamin deficiency itself, which may be exacerbated by the diversion of metabolically active forms of cobalamin into cyanocobalamin.

Acceptance of proposal (b) as the more likely mechanism would imply that most patients with tobacco amblyopia should also have other symptoms of cobalamin deficiency, e.g. megaloblastosis.

The literature, however, describes many cases of tobacco amblyopia without consistent patterns of cobalamin depletion (Beck, 1975), thus supporting proposal (a). It is likely, then, that some of the neurological symptoms occasionally seen in cobalamin deficiency can be attributed to excessive levels of cyanide, probably because of high cyanide intake coupled with a reduced ability to detoxify the substance.

### ***Excess Homocysteine or Cystathionine***

Several different congenital enzymatic defects have been discovered and identified in patients exhibiting homocystinuria. Along with mental retardation, these patients have ocular and mental abnormalities.

One of the inborn errors of metabolism which is known to cause homocystinuria is a defect in the homocysteine methyltransferase apoenzyme (reaction B). Thus, this anomaly should be similar in its effects to a cobalamin deficiency. It is not positively known, however, whether the clinical symptoms of homocystinuria are a result only of the toxic effects of excess homocysteine (or of the next sequential metabolite, cystathionine) or due to metabolic disturbances in the pathways affected by homocysteine methyltransferase. It seems likely that homocysteine alone can cause damage, for homocystinuria resulting from other enzyme lesions (i.e. not the methyltransferase enzyme) have been characterized. Again, it is difficult to separate immediate effects from wider-ranging influences on metabolic pathways caused by excess concentrations of substrates.

### ***Excess Methylmalonic Acid or Propionic Acid***

As discussed earlier, adenosylcobalamin participates in the conversion of methylmalonyl CoA to succinyl CoA. Thus, cobalamin is involved in the metabolism of odd-chain fatty acids and of the amino acids methionine, threonine, and isoleucine, all via propionyl CoA, as well as the metabolism of valine.

Depending on the importance of this pathway in a given individual (which is to a large extent related to diet), cobalamin deficiency may result in excess levels of propionic or methylmalonic acid. Such an excess leads to methylmalonic aciduria in many cobalamin deficiency patients. It has frequently been observed, however, that the degree of methylmalonic aciduria cannot be correlated with the presence or severity of neurological disease (although it has been reported that urinary excretion of acetic acid does correlate) (Cox et al, 1968). Recently, Frenkel has shown, using nerve biopsy slices from PA patients, that propionate is incorporated into fatty acids to produce branched-chain acids, while at the same time, acetate incorporation is inhibited, in comparison to normal tissue (Frenkel, 1971). Possibly, the inhibition of acetate incorporation is the cause of urinary excretion of acetic acid.

The above evidence strongly suggests that accumulated propionyl CoA is related to the neuropathy of cobalamin deficiency, by becoming incorporated into myelin lipids as branched-chain fatty acids. The lipids synthesized (as part of the normal turnover of myelin) are therefore abnormal, so that the resulting myelin is defective, and "demyelination" occurs.

This theory appears particularly attractive for explaining the clinical features of cobalamin deficiencies in strict vegetarians (for example, the Vegans in Britain). In such individuals, only neurological symptoms manifest, with no megaloblastosis. A diet in which fatty acids are derived solely from vegetable matter will have a high quantity of odd-chain-length fatty acids, which produce propionyl CoA as a metabolite. The large quantities of folate in such a diet are probably responsible for preventing megaloblastosis, as it very likely does in cobalamin-deficient sheep.



### ***Insufficient Synthesis of Phosphatidyl Choline***

As discussed above, one of the syntheses contributed to by the activated methyl cycle (S-adenosylmethionine) is the conversion of phosphatidyl ethanolamine to phosphatidyl choline, also known as lecithin. Both of these phospholipids are normal constituents of myelin.

Decreased activity in the activated methyl cycle as a result of cobalamin deficiency might be expected to cause a decrease in the synthesis of lecithin. A recent study on plasma lipid and phospholipid composition in PA patients (Wallenstein et al, 1977) supports this hypothesis. This study found that before treatment, these patients had decreased values for all phospholipid fractions measured (i.e. lysolecithin, sphingomyelin, lecithin) except for phosphatidyl ethanolamine (PE). After treatment, all phospholipid fractions returned to normal. Although it may be foolhardy to extend results obtained for plasma lipids to the CNS, it appears that the relatively high concentration of substrate (PE) signals an impaired synthesis of lecithin.

Since lecithin is one of the myelin constituents whose turnover is relatively rapid (along with phosphatidyl inositol and phosphatidyl serine) (Beck, 1975), it is possible that low lecithin concentrations (and/or high PE concentrations) may lead to defective myelin being substituted for normal myelin in the normal turnover of myelin components. Again, defective myelin would lead to demyelination.

### ***Defective Methylation of Ribosomal RNA***

According to Stryer (Stryer, 1975), the formation of ribosomal RNA in mammalian cells involves, after transcription, methylation of ribose units by S-adenosylmethionine (SAM), prior to cleavage into fragments which are constituents of mammalian ribosomes.

If the synthesis of SAM is slowed down due to a lack of cobalamin (reaction B), the formation of ribosomes may be impaired, perhaps more in some tissues than in others. Insufficient ribosomes (or perhaps defective ribosomes) may effect synthesis of proteins. In the CNS, this may result in disturbances in enzymes responsible for myelin synthesis or turnover, or possibly in other important proteins, such as membrane receptors for neurotransmitters.

This hypothesis is thus far based entirely on speculation. It may be worthwhile, however, to design experiments to determine if, for example, net protein synthesis is reduced in cobalamin-deficient cell cultures.

### ***Conclusion***

Available evidence to date neither confirms nor rules out any of the above mechanisms as the cause of the neuropathy associated with cobalamin deficiency. This author feels that more than one mechanism is likely to be at work in any given patient. The particular mechanism which predominates (if any) is probably determined by factors such as diet (e.g. diets high in exogenous cyanides, folates, or odd-chain fatty acids); smoking habits (cigarette smokers probably receive greater doses of cyanide from tobacco, because of inhaling, then pipe or cigar smokers or non-smokers); or inborn differences in metabolism of folates, cobalamin, SAM, and so on.

It seems reasonable to assume that, if different mechanisms are at work, then the clinical symptoms, histopathology, and biochemistry is likely to differ, depending on which mechanism(s) predominate. However, it should be possible to determine if correlations exist in these areas. This may be a fruitful topic for further research into the neuropathology of pernicious anemia and other cobalamin deficiencies.

Future work in this field is likely to benefit from recent experiments on rhesus monkeys, in which cobalamin deficiencies were induced by feeding defined diets under controlled conditions. These animals developed neuropathies after five years, but no hematological signs.

Upon autopsy, nervous system lesions indistinguishable from those due to cobalamin deficiency in humans were found (Agamanolis et al, 1976). This is an important development because it allows cobalamin deficiency neuropathies to be studied in an experimental animal system, instead of on PA patients, as previously.

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